Supporting Information

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SI Methods

Preparation and Characterization of Composite Substrates. Composite substrates with micropatterned islands of SU-8 photoresist grafted on the surface of polyacrylamide hydrogels were prepared as described previously (1, 2). Briefly, a coverglass was activated with 30 μL of bind-silane working solution according to the instruction of the manufacturer (GE Healthcare), to allow bonding of the polyacrylamide gel to the glass surface during polymerization. Polyacrylamide hydrogels of two different rigidities were prepared: a stiff gel of 13 kPa was prepared with a final concentration of 8% (vol/vol) acrylamide (Bio-Rad) and 0.2% bis-acrylamide (Bio-Rad), whereas a soft gel of 800 Pa was prepared with a final concentration of 3% (vol/vol) acrylamide and 0.08% bis-acrylamide (3, 4). Polymerization was induced by adding ammonium persulfate (Sigma-Aldrich) and N,N,N′,N′ tetramethylethylenediamine (Bio-Rad) to the acrylamide solution after degassing, and a 30-μL drop was pipetted onto the bind-silane activated coverslip. A 25-mm circular coverslip, spin coated with 50% wt/vol sucrose solution, was inverted onto the acrylamide drop. After 1 h of polymerization, the sucrose solution was dissolved to allow gentle removal of the circular coverslip. The gel was equilibrated in deionized water for 30 min then dehydrated overnight.

Arrays of rigid islands were grafted onto dried polyacrylamide gels using a negative photoresist SU-8 2000 (MicroChem). Briefly, coverslips with dried hydrogel were baked at 95 °C for 1 min and cooled to room temperature before coating with 300 μL of SU-8 using a spin coater at 5000 rpm for 20 s (WS-650s-6NPP/Lite Spin Coater, Laurell Technologies, North Wales, PA). The coverslips were baked for 3 min, cooled to room temperature, then exposed to UV light (360 nm, 100 $mJ \cdot cm^{-2}$) underneath a photomask with designed pattern (Photo Sciences) for 90 s, then baked for another 3 min at 95 °C before immersion in the SU-8 developer (MicroChem) for 90 s to generate the pattern (Fig. 1*A*). Developed coverslips were rinsed twice with 95% ethanol, then baked at 95 °C for 4 h to ensure removal of any residual developer. Before use, the hydrogel was rehydrated in PBS for 1 h.

To quantify the relative amount of matrix protein adsorbed on SU-8, Alexa Fluor 546 (Life Technologies) conjugated fibronectin (Sigma) or gelatin (Sigma) was prepared according to manufacturer instructions. The 10 μg/mL fibronectin solution or 0.1% gelatin solution was incubated on control or testing substrates for 30 min. The substrates were rinsed three times with PBS, and fluorescence images of the islands were detected using a Zeiss Axiovert 200 M with a 40× PlanFluor 0.75 N.A. dry objective lens. Fluorescence intensity was quantified using ImageJ. To measure the height of SU-8 islands, the autofluorescence of SU-8 was used for optical sectioning and *Z* stacks of 0.5-μm slices were collected using a Zeiss LSM 700 confocal microscope with a 63× Plan-Apo 1.40 N.A. oil immersion objective lens. The height of orthogonal projection of the stacks was measured using ImageJ.

Cell Culture and Phamacological Treatments. NIH 3T3 cells (ATCC) were maintained in DMEM (Life Technologies) supplemented with 10% (vol/vol) adult donor bovine serum (Thermo Scientific), 2 mM L-glutamine, 50 μg/mL streptomycin, and 50 units/mL penicillin (Life Technologies). To inhibit myosin contractility, cells were treated with 10 μM blebbistatin (Calbiochem) for either 30 min before the microneedle experiment or 16 h before quantitative analysis of pattern occupancy. For inhibition studies, cells were treated with 10 μM ML-141 to inhibit Cdc42 (Tocris) or 100 μM NSC23766 to inhibit Rac1 (Tocris) for either 30 min before starting time-lapse imaging or 16 h before quantitative analysis of pattern occupancy. The number of cells analyzed for each graph is indicated in the figure caption, and at least three independent trials were performed for each experiment.

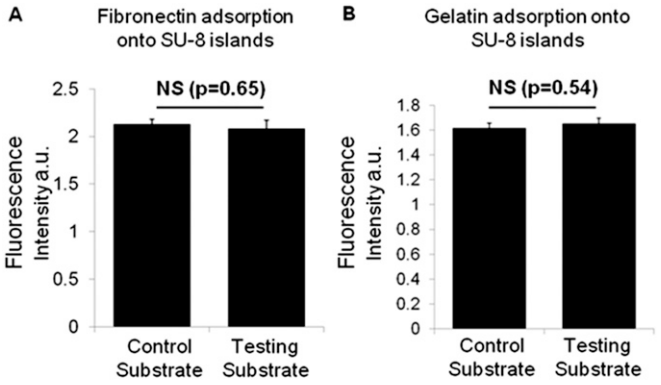
Fixation and Fluorescent Labeling. For visualizing focal adhesions, cells seeded on patterned substrates were fixed 16 h after plating with 4% (vol/vol) formaldehyde [from 16% (vol/vol) stock; Thermo Scientific] and stained with antibodies against paxillin (Santa Cruz Biotechnology) and Alexa Fluor 546 goat anti-rabbit IgG secondary antibodies (Life Technologies).

For visualizing actin filaments, cells seeded on patterned substrates were fixed 7 h after plating with 0.5% gluteraldehyde (Sigma-Aldrich) with 0.2% Triton-X 100 for 1 min, then 1% gluteraldehyde for 15 min. Samples were incubated with fresh 0.5 mg/mL $NaBH_4$ for 5 min and rinsed with PBS before staining with rhodamine-labeled phalloidin (Molecular Probes) for 30 min according to the manufacturer's instructions.

Microscopy and Image Analysis. Phase contrast images were collected with a Nikon Eclipse Ti microscope using a 40× PlanFluor 0.75 N.A. dry objective. For time-lapse videos, images were collected every 10 min for a period of 16 h. For the analysis of pattern occupancy, images were collected after 16 h of incubation. In all experiments, only single cells were counted for the analysis. For micromanipulation, a microneedle prepared with a vertical micropipette puller (model 720; David Kopf Instruments) was mounted on a Leitz micromanipulator to allow precise positioning of the tip. Phase contrast images were collected with a Zeiss Axiovert 200 M using a 40× PlanNeofluar 0.75 N.A. dry objective. Images were collected every minute during microneedle manipulation. Fluorescence images were collected using a 100× PlanFluor 1.3 N.A. oil immersion lens. Focal adhesion size was quantified using ImageJ. Contrast was enhanced to reveal the very dim actin structures in thin protrusions in Fig. 3 *A* and *C*.

Scanning Electron Microscopy. Cells on control substrates (with rigid underlying gels) were fixed 3 h after plating, when cells start to occupy small islands, with 2% (vol/vol) paraformaldehyde and 2% (vol/vol) glutaraldehyde in PBS. Scanning electron microscopy was performed at the Electron Microscopy Facility at Carnegie Mellon University.

1. Hoffecker IT, Guo WH, Wang YL (2011) Assessing the spatial resolution of cellular rigidity sensing using a micropatterned hydrogel-photoresist composite. *Lab Chip* 11(20):3538–3544.
2. Wong S, Guo W, Hoffecker IT, Wang Y (2014) Preparation of a micropatterned rigid-soft composite substrate for probing cellular rigidity sensing. *Methods in Cell Biology* 121:3–15.
3. Stroka KM, Aranda-Espinoza H (2009) Neutrophils display biphasic relationship between migration and substrate stiffness. *Cell Motil Cytoskeleton* 66(6):328–341.
4. Flanagan LA, Ju YE, Marg B, Osterfield M, Janmey PA (2002) Neurite branching on deformable substrates. *Neuroreport* 13(18):2411–2415.



	Average SU-8 Thickness (μm)
Control Substrate	2.8 ± 0.2 (n = 29)
Testing Substrate	2.6 ± 0.4 (n = 29)
p-value	p = 0.10

Fig. S1. Adsorption of fluorescently tagged fibronectin (*A*) or gelatin (*B*) to SU-8 islands is unaffected by the stiffness of the underlying hydrogel ($n = 30$ for both). In addition, there is no difference in the average height of SU-8 islands on control and testing substrates, measured using laser confocal microscopy. Error bars represent SEM.

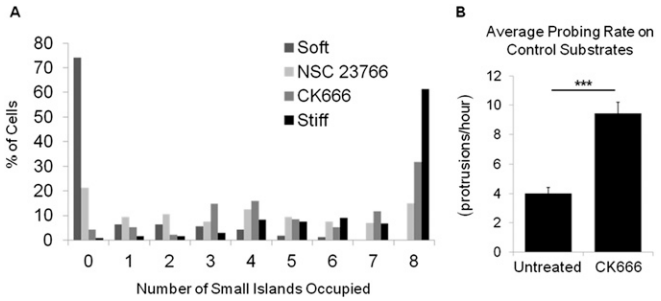
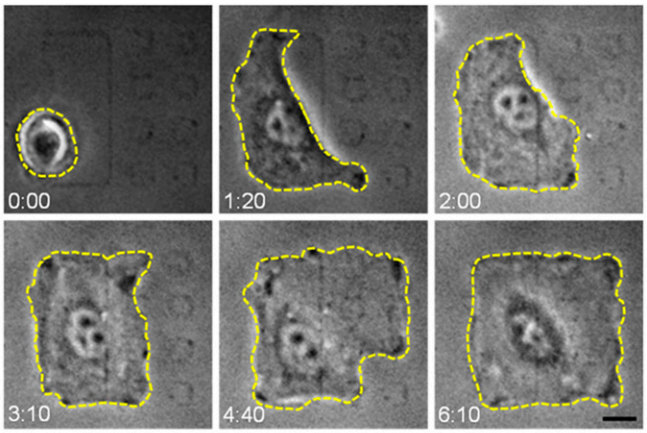


Fig. S2. Histogram showing the distribution of the number of small islands occupied by NIH 3T3 cells on control (rigid hydrogels; *A*, "Stiff" in graph, $n = 132$) or testing (soft hydrogels; *A*, "Soft" in graph, $n = 233$) substrates. Inhibition of Rac1 with 100 μM NSC23766 or Arp 2/3 with 25 μM CK666 reduced the extent of spreading on control substrates (*A*, "NSC23766" and "CK666" in graph, $N = 160$ and 94, respectively). Inhibition of Arp 2/3 also increased the rate of probing on control substrates relative to untreated cells (*B*, $N = 36$ and 22, respectively). Error bars represent SEM $^{***}P < 0.001$.



	Control Substrate	Patterned Gel	p value
Time to fully spread over pattern area (hrs)	8.0 ± 0.4 (n = 48)	8.7 ± 0.5 (n = 47)	p = 0.26
Average # Small Islands Occupied	6.8 ± 0.2 (n = 129)	6.4 ± 0.2 (n = 132)	p = 0.12
Spreading Area (um^2)	1667 ± 25 (n = 129)	1650 ± 27 (n = 132)	p = 0.64

Fig. S3. NIH 3T3 cells display similar spreading behavior on composite control substrates and on uniformly rigid substrates. Uniformly rigid polyacrylamide gels prepared with 8% acrylamide and 0.2% bis-acrylamide were conjugated with gelatin in the same island pattern as for the composite substrates. Time-lapse phase contrast microscopy indicates that cells spread to cover all of the islands following a time course similar to that for cells on composite control substrates. Numbers at the bottom of each image indicate the lapse time in hours and minutes after initial cell attachment to the large island and cell outlines are indicated by yellow dashed lines. Other aspects of the spreading kinetics are also similar between composite and homogeneous substrates with the island pattern with no statistical differences found. (Scale bar, 10 μm.)

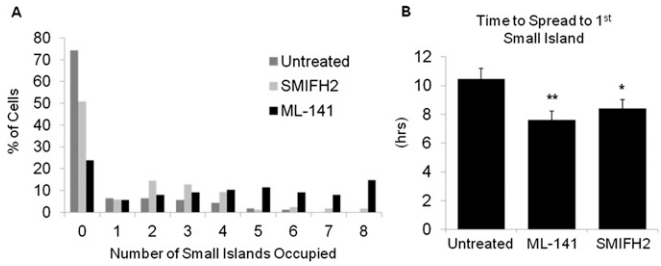


Fig. S4. Inhibition of molecules involved with filopodia function, 10 μM ML-141 to inhibit Cdc42 or 10 μM SMIFH2 to inhibit formins increases the percentage of fibroblasts that cross the rigidity border on testing substrates (*A*, "SMIFH2" and "ML-141" in graph $n = 88$ and 173). Treated cells are also able to occupy small islands at a quicker rate than untreated cells (*B*, $n = 46$ and 35). Error bars represent SEM $^*P < 0.05$ $^{**}P < 0.01$.

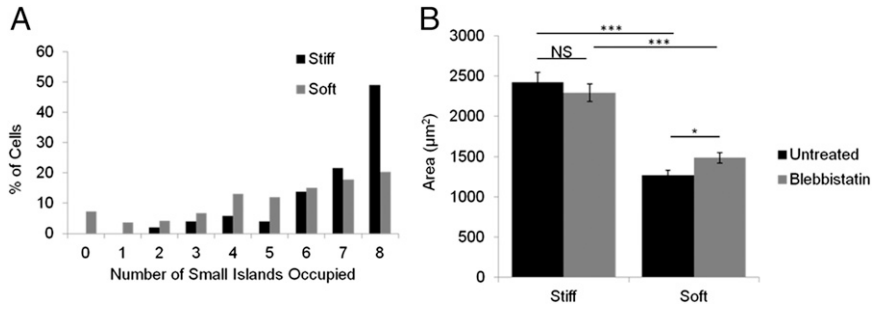
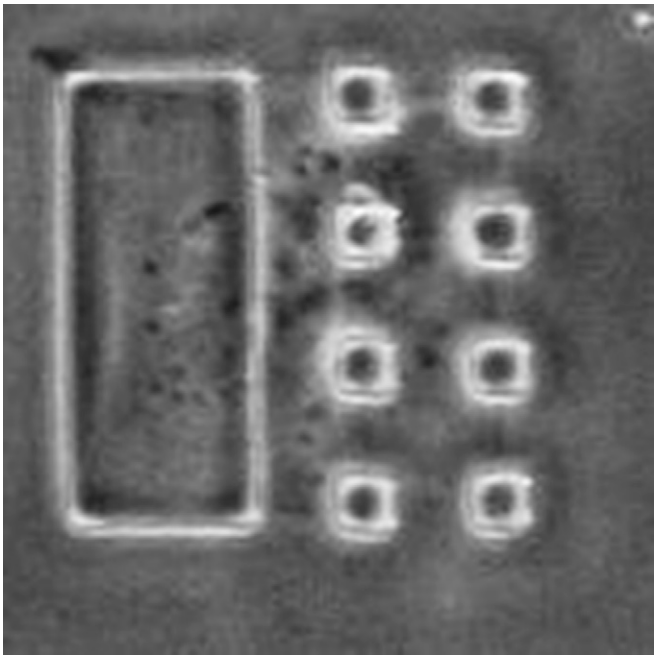
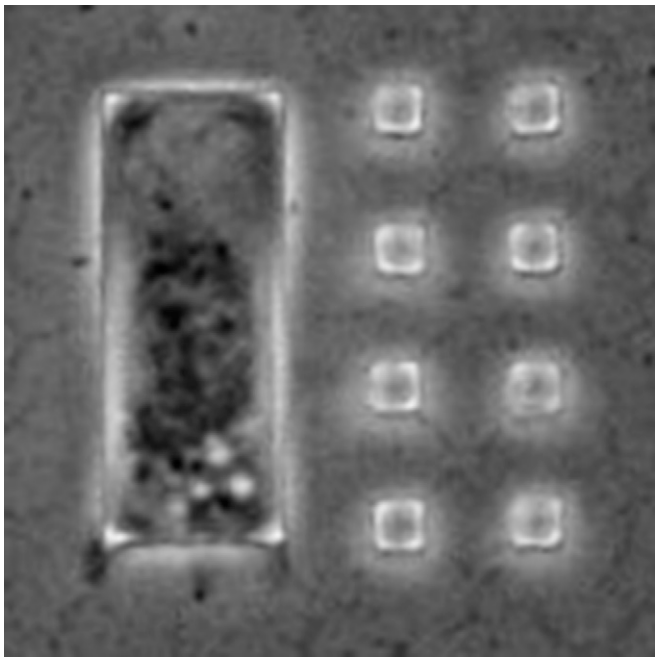


Fig. S5. Inhibition of myosin II causes impairment of rigidity sensing. Histogram showing the distribution of the number of small islands occupied show that treatment with 10 μM blebbistatin causes a large increase in the fraction of cells that occupy most of the small islands on testing substrates, compared with untreated cells (Fig. S1). However, the extent of occupancy remains lower than that on control substrates (*A*, black bars). On soft unpatterned polyacrylamide hydrogel substrates, blebbistatin-treated NIH 3T3 cells spread to a slightly, but significantly, larger area than untreated cells (*B*, right bars). There is no statistically significant difference in spreading area on stiff unpatterned polyacryalmide gels regardless of blebbistatin treatment (*B*, left bars, $n = 51$ and 52). However, the spreading area of blebbistatin-treated cells remains larger on stiff than on soft substrates (*B*, gray bars, $n = 52$ and 52). These results are similar to the results on the composite substrates in Fig. 5*B*. Error bars represent SEM $***P < 0.001$ $*P < 0.05$.



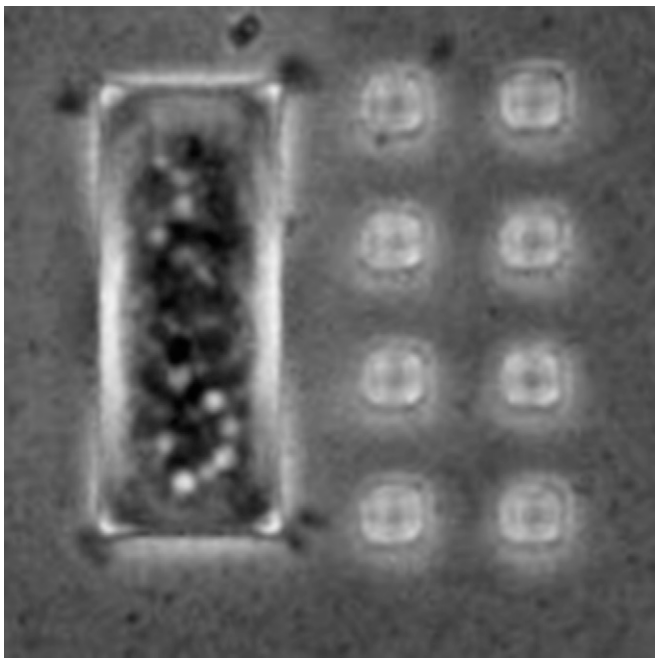
Movie S1. Time-lapse movie of a cell spreading on control substrate, recorded every 10 min. Total duration, 9.5 h.

Movie S1



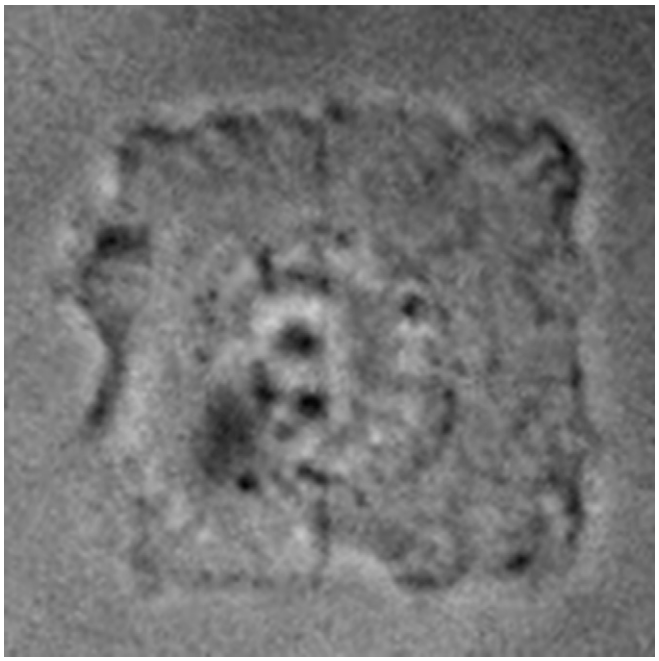
Movie S2. Defections of small islands caused by traction forces. Comparison of images recorded 10 min apart reveals minute movement of the second small island from the top just before the cell occupies that island.

Movie S2



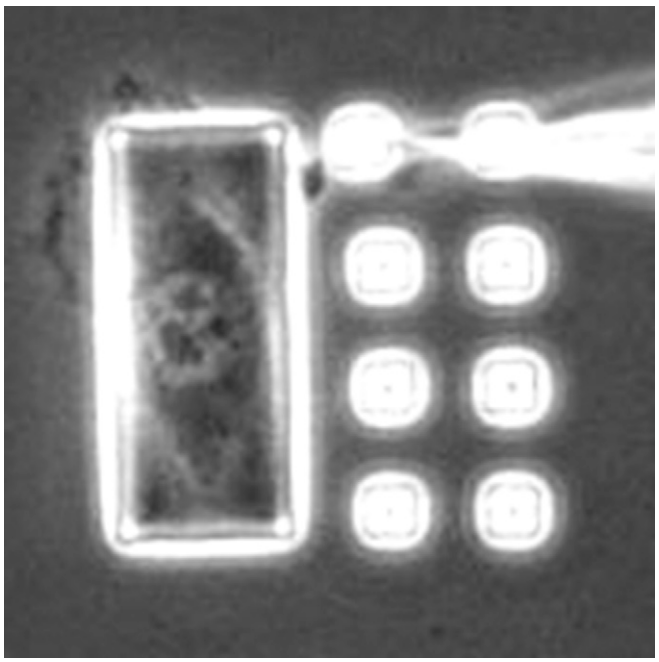
Movie S3. Time-lapse movie of a cell spreading on testing substrate, recorded every 10 min. Total duration, 14.2 h.

Movie S3



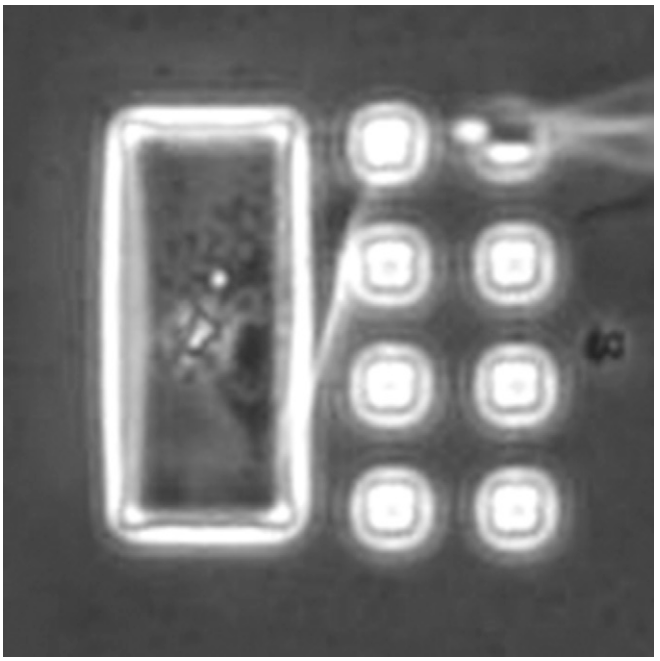
Movie S4. Time-lapse movie of a cell spreading on a homogeneous rigid polyacrylamide hydrogel, conjugated with gelatin in the same micropattern as for SU-8 islands on composite substrates. Images were captured every 10 min. Total duration, 13.2 h.

Movie S4



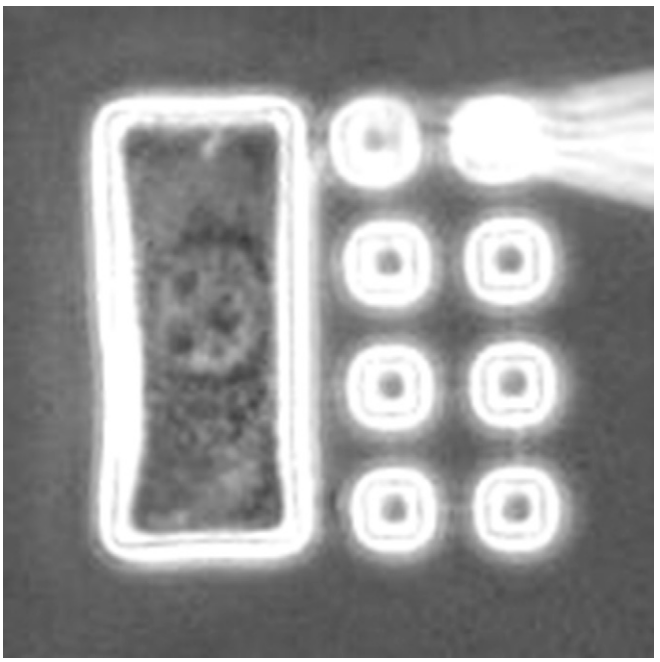
Movie S5. Time-lapse movie of a cell with a nascent protrusion on control substrates during microneedle manipulation. Images for the time-lapse movie were captured every minute. Total duration, 9 min.

Movie S5



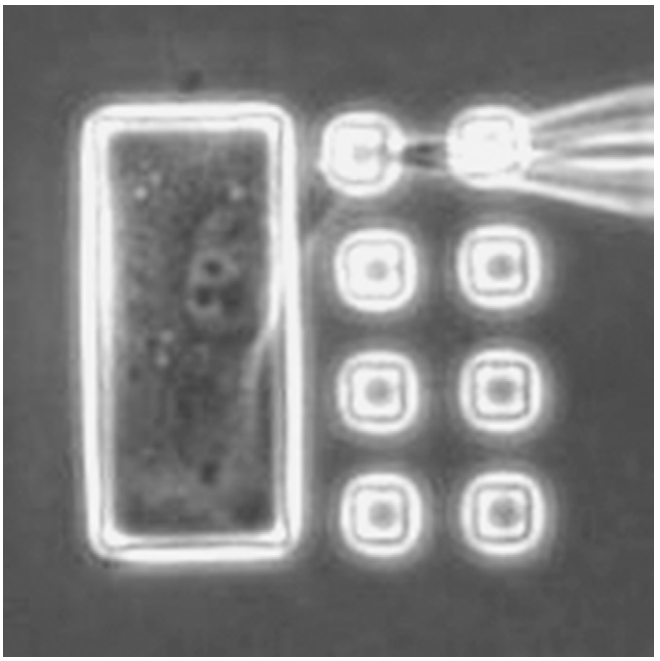
Movie S6. Time-lapse movie of a cell with a mature protrusion on control substrates during microneedle manipulation. Images for the time-lapse movie were captured every minute. Total duration, 25 min.

Movie S6



Movie S7. Time-lapse movie of a blebbistatin-treated cell with a nascent protrusion on control substrate during microneedle manipulation. Images for the time-lapse movie were captured every minute. Total duration, 8 min.

Movie S7



Movie S8. Time-lapse movie of a blebbistatin-treated cell with a mature protrusion on control substrates during microneedle manipulation. Images for the time-lapse movie were captured every minute. Total duration, 24 min.

Movie S8